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A size analysis of the adenovirus replicon

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The linear double-stranded genome of adenovirus DNA replicates semiconservatively from two origins of replication at either of the two molecular ends. Using an *in vitro* replication system which is able to initiate *de novo* DNA synthesis we have mapped the origin of DNA replication within the terminal 19 bp of the viral genome. Our conclusions are based on the use of different natural DNA templates, i.e., adenovirus type 2 and mouse adenovirus Fl DNA. In addition, we have employed linearized plasmid DNA templates which contain cloned terminal restriction enzyme fragments as well as chemically synthesized adenovirus termini of different length.
Key words: adenovirus/DNA replication/origin/chemical DNA synthesis

Introduction

The adenovirus genome is a linear DNA duplex with a length of ~36 kbp. Its two origins of replication at the molecular termini are characterized by two unique structural features, the terminal protein (TP) and the inverted terminal repetition. The development of *in vitro* replication systems (Challberg and Kelly, 1979) permitted the identification of the functional role of the TP which is covalently linked to the 5′-terminal deoxycytidine residue of the viral DNA. Its virus-coded 80-kd precursor (pTP; Smart and Stillman, 1982), which can be isolated from adenovirus type 2 (Ad2)-infected HeLa cells as a complex with the 120-kd viral DNA polymerase (Enomoto *et al.*, 1981), reacts with dCTP in an adenovirus DNA template-dependent reaction to form a covalent pTP/dCMP complex (Figure 1). This complex, in turn, provides the 3′-hydroxy primer for the elongation reaction and the subsequent formation of nascent DNA strands.

The initiation reaction, e.g., the formation of the pTP/dCMP complex, can easily be monitored by the transfer of label from [α-^{32}P]dCTP to the pTP. The observation by Tamanoi and Stillman (1982) that cloned adenovirus DNA termini which lack the TP are as efficient as genuine, virion-derived DNA termini in this initiation assay provides the possibility of defining the minimal DNA sequence required in '*cis*' for the template function.

We have used either natural adenovirus DNA/protein complexes from human and mouse adenoviruses or chemically synthesized, cloned fragments of various length from the molecular ends of Ad2 DNA to study initiation. We find that a DNA sequence as short as the terminal 19 bp but longer than 17 bp is both necessary and sufficient to initiate adenovirus-specific DNA replication *in vitro*.

Results

Initiation of DNA synthesis on natural templates

The inverted terminal repetitions of human Ad2 and mouse adenovirus Fl (AdFl) DNA which are 103 and 93 bp long, respectively, share little homology. The only conspicuous stretch of homology occurs at the very ends of the viral DNA molecules where the terminal 17 bp are identical (Figure 2). This sequence includes an A/T-rich region between positions 9 and 17 which is conserved in all known human, simian and mouse adenovirus DNA termini (Figure 2) (Stillman *et al.*, 1982). We asked whether the respective adenovirus DNA/protein complexes, with only 17 terminal base pairs in common, would be acceptable templates in the template-dependent transfer reaction using both homologous and heterologous replication systems. Nuclear extracts were prepared from Ad2-infected HeLa and AdFl-infected 3T3 cells, incubated with the different adenovirus DNA/protein complexes in the presence of α-^{32}P-labelled dCTP and analyzed by electrophoresis on SDS-polyacrylamide gels. Active templates and extracts were identified upon autoradiography by the appearance of a band at either the 80 K position (Ad2-pTP) or the 73 K position (AdFl-pTP). As shown in Figure 3, lane 2, the Ad2-derived DNA/protein complex is only active in nuclear extracts from Ad2-infected HeLa cells but not in extracts from AdFl-infected 3T3 cells (Figure 3, lane 7). Likewise, the AdFl DNA/protein complex is only active in the homologous (Figure 3, lane 8) but not in the heterologous

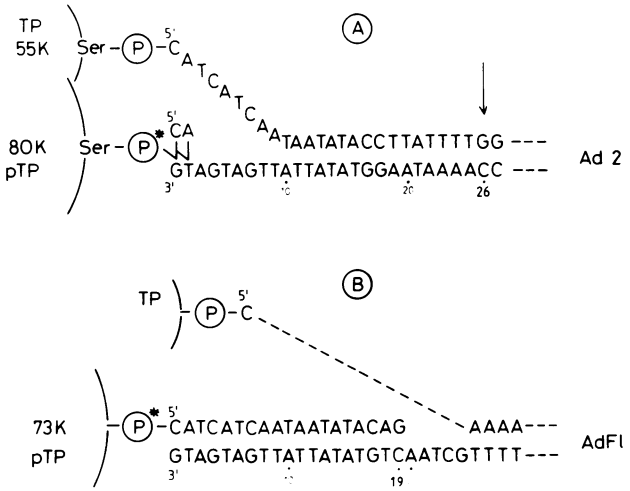

Fig. 1. Assays for initiation and limited elongation of adenovirus DNA replication. Initiation (**A**) is assayed through incubation of a proper DNA template in the presence of [α-^{32}P]dCTP and ddATP. For the elongation reaction (**B**) the incubation is performed in the presence of [α-^{32}P]dCTP, dATP, dTTP and ddGTP. Replication under these conditions will proceed only towards the first dG residue. In Ad2 DNA, the template in (**A**), this is position 26 (vertical arrow), in AdFl DNA [the template in (**B**)] position 19. In both assays, the ^{32}P-labelled pTP is identified through polyacrylamide-SDS gel electrophoresis.

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                    10        20        30
Ad2-      CATCATCAATAATATACCTTATTTTGGATT---  103
Ad2-      CTATCTATATAATATACCTTATTTTGGATT---  103
Ad4-      CTATCTATATAATATACCTTATTTTTTTTG---  116
Ad7-      CTATCTATATAATATACCTTATAGATGGAA---  136
Ad12-     CTATATATATAATATACCTTATACTGGACT---  164
Ad18-     CATCATCAATAATATACCTTATACTGGACT---  162
AdFl (MOUSE) CATCATCAATAATATACAGTTAGCAAAAAA---  93
CELO (CHICKEN) GATGATGTATAATAACCTCAAAAACTAACG---  63
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Fig. 2. Terminal sequences of inverted terminal repetitions from different adenovirus DNA molecules. The different numbers describe the length (in bp) of the various repetitions. A region of homology is marked in bold letters. The sequences are from Stillman *et al.* (1982) for the human adenoviruses Ad2 to Ad18, from Temple *et al.* (1981) for mouse AdFl and from Alestrom *et al.* (1982) for CELO-virus.

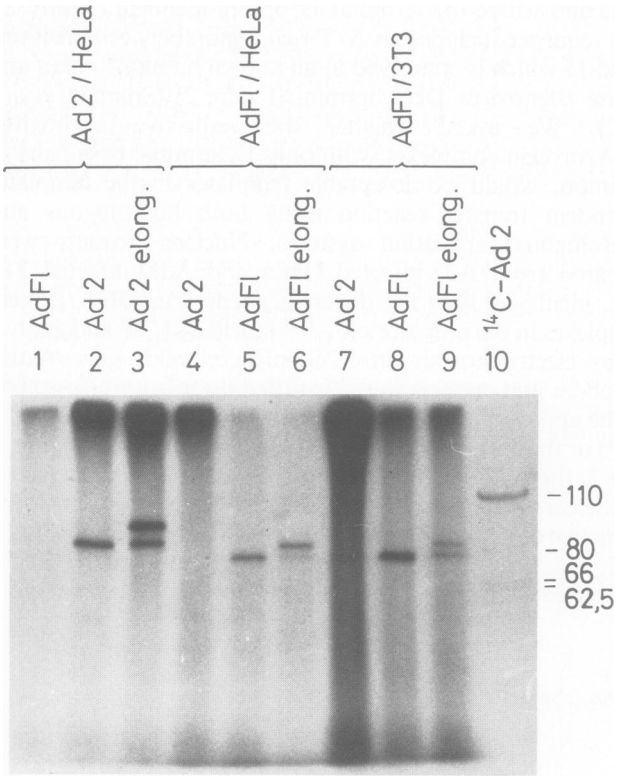

Fig. 3. Initiation and limited elongation assays with genuine Ad2 and AdFl DNA/protein complexes as templates. The autoradiogram of the 10% polyacrylamide-SDS gel shows in **lane 10** a [^{14}C]adenovirus structural protein marker with the mol. wts. indicated in kd. **Lanes 1, 2** and **3**: nuclear extracts from Ad2-infected HeLa cells with AdFl DNA/protein complex **(lane 1)** or Ad2 DNA/protein complex **(lane 2)** in an initiation assay and Ad2 DNA/protein complex in an elongation assay **(lane 3)**. **Lanes 4, 5** and **6**: nuclear extracts from AdFl-infected HeLa cells with Ad2 DNA/protein complex **(lane 4)** and AdFl DNA/protein complex **(lane 5)** in initiation assays and AdFl DNA/protein complex in an elongation assay **(lane 6)**. **Lanes 7, 8,** and **9**: extracts from AdFl-infected 3T3 cells with Ad2 DNA/protein complex **(lane 7)** and AdFl DNA/protein complex **(lane 8)** in initiation assays and AdFl DNA/protein complex in an elongation assay.

system (Figure 3, lane 1). As already indicated above, the [^{32}P]dCMP transfer in the mouse system occurs to a protein which is ~6–8 kd smaller than the Ad2-derived precursor to the terminal protein. It is assumed in this paper that this 73-K protein represents the virus-coded pTP of AdFl virus although no additional evidence is available on this point.

Figure 3 also demonstrates that the homologous replication systems not only catalyze the template-dependent initiation,

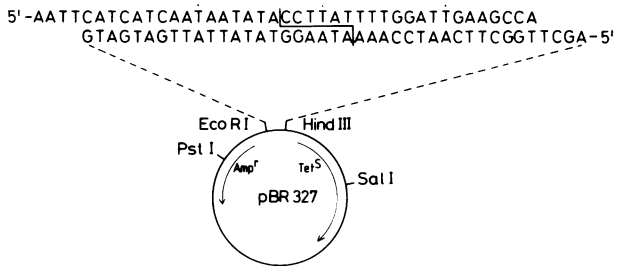

Fig. 4. Construction of the plasmid pTD38. The Ad2-specific insert was formed from four oligonucleotides as shown in the top lines and described in Materials and methods. After reannealing, the insert was cloned into a purified, *Eco*RI- and *Hind*III-linearized pBR327 DNA vector molecule. Recombinant clones were selected for ampicillin resistance/tetracycline sensitivity.

but also a limited elongation reaction. In the presence of dATP, dCTP, dTTP and ddGTP, the reaction proceeds until it reaches the first dG residue at position 26 in Ad2 and position 19 in AdFl DNA. The addition of these short oligonucleotide stretches raises the apparent mol. wt. of the pTP proteins to 88 and 78 K, respectively. This, in turn, results in the appearance of an additional band upon autoradiography, as shown in Figure 3, lane 3 for the Ad2 system, and in Figure 3, lane 9, for the AdFl system.

The observed selectivity of the nuclear extracts for their respective templates was also observed in a nuclear extract obtained from AdFl-infected HeLa cells. AdFl only grows to very low titers in HeLa cells but replicates its DNA very efficiently in this system (Antoine *et al.*, 1982). Since, in contrast to mouse 3T3 cells, HeLa cells can be grown in suspension cultures, AdFl-infected HeLa cells represent a very convenient system for the study of AdFl DNA replication. Nuclear extracts thus replicate the AdFl DNA/protein complex [Figure 3, lanes 5 (initiation) and 6 (elongation)] while they fail on the Ad2 DNA/protein complex (Figure 3, lane 4). The failure of genuine, virion-derived adenovirus DNA/protein complexes to be replicated in heterologous systems could indicate that the 17-bp homology is not sufficient to support the initiation reaction in '*cis*'. However, the two DNA probes are also distinguished by the presence of a different TP on the molecular ends. In the first round of displacement synthesis, for example, interaction of an Ad2-derived TP on the Ad2 DNA/protein complex with an AdFl-derived pTP, present in the AdF1-infected 3T3 cell nuclear extract, might be abortive and could disturb the necessary interactions with the origin of replication. In later rounds of replication or on protein-free templates these deleterious, heterologous interactions might be irrelevant.

Initiation on protein-free templates

Tamanoi and Stillman (1982) and van Bergen *et al.* (1983) have shown that plasmid DNA molecules containing terminal Ad2-specific DNA sequences can serve as templates in the initiation-specific transfer reaction of adenovirus DNA replication. Using chemical DNA synthesis we have prepared sets of oligonucleotides which represent either the 17-bp, 19-bp, 21-bp or 38-bp long terminal DNA sequences of Ad2 DNA. For cloning in the plasmid vector pBR327 (Soberon *et al.*, 1980), these fragments are flanked by *Eco*RI- and *Hind*III-specific, 5'-overhanging DNA stretches (Figure 4). Linearization of the purified plasmids pTD17, pTD19, pTD21 and pTD38 with *Eco*RI thus yields linear DNA

Table I. Structures and biological activities of DNA-specific termini in linearized plasmid DNA molecules

			Ad 2 HeLa (NE)
pTD 17	(pBR 327)	5'-AATTCATCATCAATAATATAC------ (17) 3'- GTAGTAGTTATTATATG------	-
pTD 19/21	(pBR 327)	5'-AATTCATCATCAATAATATACCTTA------ (19, 21) 3'- GTAGTAGTTATTATATGGAAT------	+
pTD 38	(pBR 327)	5'-AATTCATCATCAATAATATACCTT------CAA--- (20, 38) 3'- GTAGTAGTTATTATATGGAA------GTT---	+
pComa I	(pUC 8)	5'-AATTCATCATCAATAATATACCTT------ACA--- (20, 1003) 3'- GTAGTAGTTATTATATGGAA------TGT---	+
pGA	(pUC 8)	5'-AATTCCCCATCATCAATAATATACAGT--------G--- (20, PstI) 3'- GGGGTAGTAGTTATTATATGTCA--------C---	-

Plasmids pTD17, pTD19, pTD21 and pTD38 were constructed as indicated in Figure 4 and described in Materials and methods. The plasmid pComaI contains the right-hand terminal *Hind*III-K fragment (1003 bp) from Ad2 DNA and plasmid pGA the right-hand terminal *Pst*I-H fragment (~2600 bp) from AdFl DNA. The (+) and (−) signs indicate the presence or absence of template activity of the various plasmid DNA molecules in the initiation and/or the limited elongation assay as performed with extracts from Ad2-infected HeLa cells.

molecules with the terminal Ad2-specific sequences at one of their termini (Table I). In addition, the right-hand terminal *Hind*III-K fragment of Ad2 and the right-hand terminal *Pst*I fragment of AdFl DNA were cloned in the vector pUC8 (Vieira and Messing, 1982). The 1008 bp long Ad2 *Hind*III-K fragment was isolated from a *Hind*III digest of wild-type Ad2 DNA and treated with alkali to remove the terminal protein. After reassociation, the DNA fragment was cloned into linearized pUC8 DNA containing a filled-in *Eco*RI site and a normal *Hind*III terminus. The resulting plasmid pComaI (Table I) can be linearized to yield one Ad2-specific terminus flanked by the 5'-overhanging *Eco*RI sequences. The terminal AdFl DNA *Pst*I fragment was prepared similarly but cloned into a linearized pUC8 DNA containing a blunt end *Sma*I and a normal *Pst*I terminus. Upon linearization with *Eco*RI, the plasmid pGA thus yields an AdFl-specific terminus with three additional C/G base pairs in addition to the 5'-overhanging *Eco*RI terminus (Table I).

The results of the initiation and the limited elongation assays with these plasmids, using extracts from Ad2-infected HeLa cells, are documented in Figure 5 and summarized in Table I. The plasmid-derived templates containing 1003- and 38-bp terminal sequences from Ad2 DNA show similar activity (Figure 5, lanes 3 and 7), both qualitatively and quantitatively, to the genuine Ad2 DNA/protein complex (Figure 6, lane 1). In addition, these plasmids are indistinguishable from the Ad2 DNA/protein complex in the limited elongation assay (Figure 5, lanes 2, 4 and 8). The linearized plasmids pTD19 and pTD21 are clearly active in the initiation assay (Figure 5, lanes 11 and 12) but yield only one third of the activity of pTD38. The plasmid pTD17 with the 17 bp long terminus (Figure 5, lane 10) and the plasmid pGA with AdFl-specific sequences (not shown) are totally inactive in the extracts derived from Ad2-infected HeLa cells. Experiments with supercoiled and linear forms of the parental plasmids pBR327 (Figures 5, lane 9) and pUC8 (not shown) proved to be negative. In the case of pTD17, even a 100-fold increase in DNA termini concentration did not reveal any activity in the

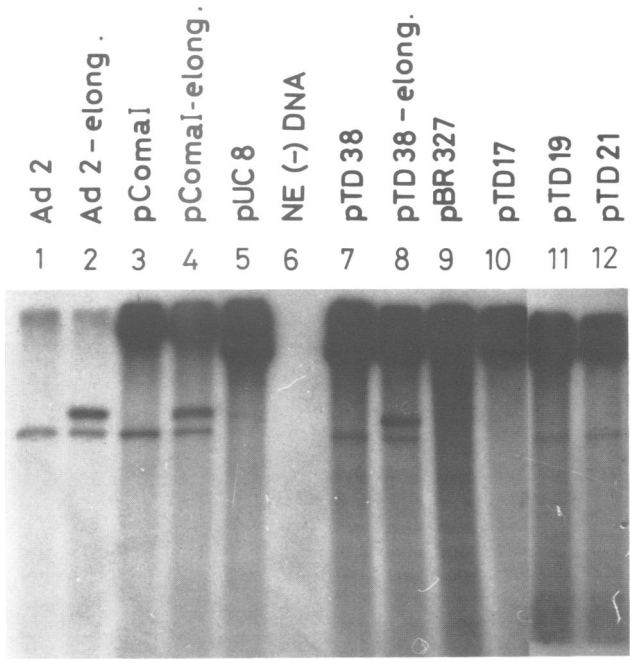

Fig. 5. Initiation and limited elongation assays with adenovirus termini containing plasmid DNA molecules. Nuclear extracts were derived from Ad2-infected HeLa cells. **Lanes 1, 3, 7, 10, 11** and **12**: initiation assays with the genuine Ad2 DNA/protein complex, pComaI, pTD38, pTD17, pTD19 and pTD21, respectively; **lanes 2, 4** and **8**: limmited elongation asays with the genuine Ad2 DNA/protein complex, pComaI and pTD38, respectively; **lanes 5** and **9**: *Eco*RI-linearized pUC8 and supercoiled pBR327 DNA as controls; **lane 6**: nuclear extract from Ad2-infected HeLa cells in the absence of exogenously added DNA.

initiation and the elongation assay. Furthermore, pTD17 did not act as a template in the presence of partially purified nuclear extracts obtained through ammonium sulfate precipitation or DNA agarose chromatography. As a control it was demonstrated (Figure 5, lane 6) that the nuclear extracts show no endogenous activity.

Discussion

A variety of experimental approaches have previously located the origin of adenovirus DNA replication towards the molecular ends of this double-stranded DNA genome (reviewed by Winnacker, 1978; Fütterer and Winnacker, 1984). The experiments presented here define a minimal region of between 17 and 19 bp from the molecular end which can act *in vitro* as template for the initiation reaction of adenovirus DNA replication. The observed minimal origin region includes an A/T-rich stretch between positions 9 and 17, which is conserved in all human adenovirus DNA molecules, in simian adenovirus and in mouse adenovirus Fl. It can be speculated that it is this stretch of the viral DNA which presents the functional origin of adenovirus DNA replication and which acts as recognition and/or binding site for the various factors of viral and cellular origin, involved in the initiation reaction.

Our conclusions for a minimal size of 18 or 19 bp for the adenovirus replicon are based on two experimental approaches. For one, genuine adenovirus DNA/protein complexes from Ad2 and AdFl virions are active only in their homologous *in vitro* replication systems; e.g., the Ad2 DNA/protein complex in extracts from Ad2-infected HeLa cells, the AdFl DNA/protein complex in extracts from AdFl-

infected 3T3 or HeLa cells. In the respective heterologous systems, they are totally inactive. Since they only share the 17 terminal base pairs it could be argued that this sequence is not sufficient for the required template function. As pointed out above, this line of reasoning ignores the presence of different terminal proteins on these templates. We have previously shown that Ad2 and AdFl DNA show very little homology (Antoine *et al.*, 1982). It is thus to be expected that the primary sequences of these two proteins, which, as demonstrated here, are distinguished by a size difference of ~8 kd, are very different from each other. However, in view of the results with the linearized plasmid DNA molecules containing adenovirus terminal sequences without TPs, this argument appears of little significance. It is therefore definitely the differences in sequence which does not allow the Ad2 and the AdFl DNA/protein complexes to replicate in heterologous replication systems.

These conclusions are in agreement with those derived from the work of Tamanoi and Stillman (1983; and in preparation). These authors have described deletions which retain only 20 bp from the adenovirus terminal sequence and which support the initiation reaction albeit at a reduced rate. If, through our experiments, the origin of adenovirus DNA replication has now been minimized to a size of 18–19 bp, the question remains as to the other possible functions of the inverted terminal repetition. These sequences, in fact, are between 63 (CELO-virus) and 162 (Ad12) bp long (Figure 2). It will thus be necessary to ask whether the deletions are also active *in vivo* and whether a complete replication cycle might not require the presence of the full length terminal repeat. Furthermore, it might be investigated whether the extent of the observed *in vitro* initiation process might not depend on the DNA sequences neighbouring the shortened adenovirus terminus. In this context, however, it should be kept in mind that bacteriophage ϕ29 DNA, which replicates by a mechanism similar to adenovirus DNA, contains inverted repeats of only 6 bp (Yoshikawa *et al.*, 1981). Finally, we would like to point out that supercoiled plasmid DNA is totally inactive in the TP labelling assay using crude or only partially purified nuclear extracts. This is in contrast to the work of Pearson *et al.* (1983) and Enns *et al.* (1983) who have described the occurrence of rolling circles with single-stranded tails following incubation in nuclear extracts from Ad2-infected HeLa cells. However, these molecules which arise in a proportion of only 9% of the input supercoiled plasmid molecules, might be formed by a mechanism different from the initiation mechanism on linear DNA which may not involve a covalent attachment of the TP to the terminal dC residue. Both experimental approaches eventually will have to be reconciled. In any case, these data place the origin of adenovirus RNA replication within the terminal 20 bp of the viral DNA molecule.

We have obtained preliminary data on the behaviour of linearized plasmid DNA molecules containing adenovirus termini in nuclear extracts from AdFl-infected cells. In extracts from 3T3 cells, both the genuine AdFl DNA/protein complex and the linearized plasmid pGA containing the AdFl terminus show strong activity in the transfer assay and in the limited elongation test. The plasmids pTD17 and pTD38 as well as *Eco*RI-linearized pBR322 and pUC8 are active only in the initiation but not in the limited elongation assay. In AdFl-infected HeLa cells, only the genuine complex and the linearized plasmid pGA, which both contain AdFl-specific termini, are active in both assays. No activity can be detected with the plasmids pTD17 and pTD38 containing deleted Ad2 termini. The preliminary conclusion can be drawn, that the AdFl-infected 3T3 cell extracts are considerably more permissive with regard to the transfer assay than the AdFl-infected HeLa cells. Since the virus is identical in both cases, the specificity for the proper template must be conferred to the system by host factors. An analysis of these factors is underway.

Materials and methods

Cells and viruses

The origin of both HeLa and 3T3 cells has been described previously (Antoine *et al.*, 1982). Infections of mouse 3T3 cells and of HeLa cells with mouse AdFl were performed under conditions discussed by Antoine *et al.* (1982).

Preparation of nuclear extracts

Nuclear extracts both from Ad2-infected HeLa cells and AdFl-infected HeLa or 3T3 cells were prepared as described by Challberg and Kelly (1979) with the modifications of Reiter *et al.*, (1980). It proved essential to perform the infections at multiplicities of infection of at least 100 p.f.u./cell. Ad2-infected HeLa cells were harvested 20–22 h post-infection (h.p.i.) following addition of 10 mM hydroxyurea (Serva, Heidelberg) at 2 h.p.i. Extracts from AdFl-infected 3T3 cells were prepared at 40–45 h.p.i. In this case only 5 mM hydroxyurea was added at 7 h.p.i. Freshly prepared extracts were occasionally purified by precipitation with 40% ammonium sulfate. The precipitated proteins were dissolved in assay buffer and dialyzed against the same buffer in the presence of 10% sucrose. A precipitate which formed during dialysis could be removed by low speed centrifugation at 12 000 r.p.m. for 2 min in an Eppendorf centrifuge.

Chemical synthesis of DNA

Chemical synthesis of oligonucleotides was performed according to the protocol of Dörper and Winnacker (1983) which is based on the phosphoamidite method of Beaucage and Caruthers (1981). Oligonucleotides were purified by electrophoresis on 20% polyacrylamide/8 M urea gels. Syntheses were performed on a 1 μmol scale in an Applied Biosystems Type 380A DNA Synthesizer. For cloning, ~6 μg (1 nmol) each of the complementary DNA fragments (Figure 4) were incubated in 25 μl of DNA ligase buffer [66 mM Tris-Cl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM dithiothreitol (DTT)] at 70°C for 30 min and left to cool down to room temperature for 5 h. After addition of 2 μg dephosphorylated pBR327 DNA, T4 DNA ligase (3 units; New England Biolabs) and ATP (1 mM) the reaction mixture was incubated for 15 h at 6°C. Transformations into *Escherichia coli* strain HB101 (Mandel and Higa, 1970) and selection for ampicillin resistance/tetracycline sensitivity yielded ~500 clones. One representative clone was chosen from each of the four constructions and named pTD17, pTD19, pTD21 and pTD38, respectively. DNA sequences of the cloned inserts (Figure 4 and Table I) were determined as described according to Maxam and Gilbert (1980).

Preparation of adenovirus DNA/protein complexes

Adenovirus DNA/protein complexes were prepared from virions according to Sharp *et al.* (1976) with the modifications of Stillman (1981) by lysis in 4 M guanidinium hydrochloride (GuHCl) and purification through sucrose gradients containing 5–20% sucrose in 4 M GuHCl. Centrifugation was performed in a Sorvall ST 41.14 rotor at 30 000 r.p.m. and 4°C for 16 h. Peak fractions were dialysed against several changes of 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride. The dialyzed samples could be stored at 4°C for at least 1 month. In the preparation of cloning experiments, the TP was removed from the DNA/protein complex or from the terminal restriction enzyme fragments by incubation in 0.3 M NaOH for 120 min at room temperature.

Initiation and elongation assay

Reaction mixtures (25 μl) contained 25 mM Hepes/KOH (pH 7.5), 3 mM ATP, 2 mM DTT, 5 mM $MgCl_2$, 50 μM ddATP, 0.6 μM dCTP, 2 μCi [α-^{32}P]dCTP (Amersham: ~3000 Ci/mmol), 0.5–2 μg plasmid DNA or 100 ng DNA/protein complex and 8–12 μl nuclear extract (8–15 mg protein/ml). For the formation of the elongated product, ddATP was omitted and replaced by dATP, dTTP and ddGTP, each at a final concentration of 40 μM.

After incubation for 60 min at 30°C the reaction was stopped by addition of sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 3% β-mercaptoethanol, 3% SDS). Samples were heated for 3 min at 100°C and separated by electrophoresis in 10% polyacrylamide-SDS gels. ^{14}C-Labelled adenovirus was used as a mol. wt. marker.

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